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SEMI-ANNUAL PROGRESS REPORT

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MARINE BORER PROJECT

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1. Summary

As a result of accelerated leaching tests carried out over the past year, an improved protocol for further testing and estimation of the service life of creosote treatments has been drawn up. The fractions #4, #5, #6 and the residue have been tested and are now being evaluated. Field tests of heavier panels have been planned for comparison with the thin panels used in the accelerated leaching tests.

Plans have been completed for measuring the rate at which creosote diffuses from treated timbers when immersed in seawater.

During the period covered by this report, a respirometer method suitable for routine, large-scale screening of creosote fractions for toxicity to limnoria has been developed and tested. Preliminary results indicate that the "tar acid" fraction are extremely toxic.

Further observations have been made on the activity and distribution of the cellulase enzyme system of Teredo. It is definitely present in the larvae at the time of penetration of the wood. Methods have been developed for the preliminary extraction and concentration of this activity. The biochemistry of this enzyme system is being actively compared with that produced by a cellulytic mold Myrothecium verrucaria. It has been shown that the pH optima for the two systems are similar.

Studies of carbohydrate metabolism have been continued and have shown the following intermediates to be present: adenylic acid, adenosine polyphosphates, hexose phosphates and arginine phosphate.

A study of the histological changes in the larva has been initiated.

A study on growth rate of individual teredids in relation to age and breeding has been completed and is being prepared as a technical report.

A series of tests were conducted which indicated that the intensity of borer attack is independent of the length of time or conditions under which the wood has been water-soaked prior to exposure.

The degree to which creosoted wood protects adjacent wood as a result of toxic diffusion was tested by means of a new series of panels. The results showed that creosote is active only to a small degree within 1/8 inch of the edge of the treated wood, whereas antifouling paints containing copper oxide were effective at distances of more than ½ inch.

Chlorinated creosote panels provided by NRL were continued on test.

Greenheart panels at the end of three years exposure in Miami harbor showed light attack by both teredids and limnoria. Surinam timber panels at the end of 2½ years exposure showed light to heavy attack, except in the case of Anaura, which was only superficially attacked.

Improved facilities for the research have been provided at the new Virginia Key Station, which is provided with a running seawater system.

Three technical reports were issued during the half-year.

2. Accelerated Leaching Tests:

A meeting of members of the Marine Laboratory and the Naval Research Laboratory at the University of Miami on 1-3 July, 1953 led to the following basic protocol for future evaluation work of various creosote treatments and accelerated leaching tests:

(1) Test panels shall be of Southern pine, sapwood, knot-free, low in resinous materials and of fairly uniform density; dimensions:

5" x 1½" x 1/8".

(2) Test panels with whole creosote shall be impregnated from the level of 5 lbs. per cubic foot to the level of 20 lbs. per cubic foot, aproximately 20 panels to be prepared over each 5 pound range. An attempt shall be made to spread the impregnated panels evenly over the entire 15 pound range although no effort shall be made to impregnate at any specific poundage level.

(3) Test panels prepared with fractions of creosote shall be impregnated from the level of 10 pounds per cubic foot to the level of 15 pounds per cubic foot. About 20 panels shall be prepared in this range for each fraction.

(4) An attempt shall be made to rate the creosote fractions by plotting attack as a function of treatment; the ratio of the creosote treatment in lbs/cu. ft. to fraction treatment in lbs/cu. ft. of whole creosote equivalent giving equal attack shall be computed at the middle of the fraction curve and taken as the rating of the fraction relative to whole creosote.

(5) Poundage impregnation of a panel with fraction-fortified or fraction-depleted creosote shall be stated in terms of whole creosote. For example, if whole creosote contains 10% of Fraction A and 10 grams of creosote are needed, for say, a 30 pound impregnation, then a panel impregnated to 30 pounds with the fraction A fortified material would contain 10 grams of whole creosote plus 1 gram of fraction if it is desired to double the fraction. For a fraction-depleted creosote the panel would contain 9 grams of creosote minus fraction and thus, while

containing only 9 grams, would still be impregnated to a 30 pound level with respect to everything but the fraction.

(6) Two sets of panels shall be prepared for each fraction, one to be exposed without leaching and one to be exposed after a 16 day leaching period. The 16 day leaching period is the only leaching period to be used. Exposure period shall be set tentatively for six months.

(7) One set of whole creosote panels shall be prepared for each two fractions tested.

(8) Each set of panels to be tested shall be given a set number.

(9) Panels being exposed shall be inspected and rated bimonthly. They shall be scraped free of fouling biweekly.

(10) All fractions of creosote which have been evaluated shall be re-evaluated by the revised procedure.

The testing of fractions #4, #5, #6 and residual material was carried out during the semi-annual period. Evaluation is now in progress.

3. Long Term Field Tests

It was unanimously agreed that long term field tests, using panels of the size 2" x 4" x 8", should be initiated as soon as practicable. Panels of this size would have a much higher volume to surface ratio and a much slower rate of natural leaching than the small panels now being used and, therefore, come nearer to reflecting real service life. They would serve to calibrate leaching tests. The preparation of the larger panels shall be started at NRL as soon as the physical equipment necessary to handle the larger panels can be assembled. The panels for the field tests shall be prepared using whole creosote and as many of

the fractions as practicable.

4. Diffusion Studies

Tentative plans were made to study the natural rate of diffusion of creosote from the interior to the exterior of a large timber (2" x 4") and, if possible, the change in composition of the creosote, if any, taking place during this process. The tentative procedure is to expose simultaneously to seawater immersion several identical pieces of uniformly impregnated timber. At monthly intervals a test panel shall be examined by shaving off a number of 1/8" increments from the surface. The creosote shall be extracted from these shavings and examined spectroscopically. The Marine Laboratory shall prepare, expose, section, and extract the timbers. The Naval Research Laboratory shall examine the extracts spectroscopically. It may not be possible to identify individual compounds but it will be possible to determine the changes in the relative amounts of the functional groups present; for example, a decrease in hydroxyl content or a change in the proportion of aromatic to aliphatic hydrocarbon.

5. Toxicity of Creosote

The last semi-annual report related preliminary work on this subject with larval Teredo in the capillary micro-respirometer. The value of the equipment is undeniable for exacting research studies. It has, however, certain shortcomings when it's use is contemplated for routine, large-scale screening operations. Thus, it is possible to study but one animal at a time; the time required for thermal equilibration often exceeds two hours which also slows down production of useful results. Finally the instrument is too sensitive for routine use. For these rea-

sons some attention was early devoted to the possibility of employing the standard Warburg respirometer. As been pointed out in previous reports, this apparatus is not suitable for oxygen uptake studies in Teredo because of the agitation which is required. However, Limnoria, in some initial and unreported experiments several years ago, had demonstrated complete tolerance of the oscillations required to effect gaseous equilibrium in the Warburg apparatus. Further investigation of this form has shown that the combined oxygen uptake by 25 adult Limnoria during a period of one hour, is of a magnitude entirely suited to this technique of measurement. It remained only to demonstrate that whole creosote produced a measureable effect upon this total oxygen consumption. A series of dilutions of whole creosote, prepared by the method we have reported previously was studied. Table 1 shows the results of this study.

It will be observed that the effect, which is real, statistically significant and reproducible, is a definite augmentation of the oxygen uptake of the experimental animals. It is thought that this added oxygen consumption is a measure of the metabolic work being done in detoxification. It has frequently been reported that Limnoria prosper in wood which has been treated with creosote. Presumably this is only possible because of the existence of some metabolic mechanism to render the creosote relatively innocuous. It is apparent from the results shown in Table 1 that the magnitude of the response is, in general, a function of the concentration of whole creosote which is present in the medium.

Having thus demonstrated the reality of the physiological reaction to creosote in Limnoria, and the utility of Limnoria as a test animal, some attention was devoted to a study of the toxicity of creosote frac-

tions supplied us by the Naval Research Laboratory.

Since whole creosote produced a maximal reaction when it was diluted 1:10,000 with sea water, it was determined to employ this same dilution in the assay of creosote fractions. This procedure admittedly does not compare the toxicity of whole creosote with the toxicity of its component fractions when these are applied alone in the concentration at which they were present in the whole sample. Information on the quantitative composition of the original creosote sample was not available to us when the tests were initiated.

Table 1

Effect of Various Agents on Oxygen Consumption by Limnoria **

Material tested	Dilution in sea water	Number of tests	Oxygen uptake*	%Increase over normal
Control determinations		47	11.935±2.83	0
M/1,000 Glucose in sea water		10	14.267	19.5
Whole Creosote	$1:10^6$	25	16.033	34.2
Whole Creosote	$1:10^4$	19	21.249±3.90	77.5
"Solvent Extraction fraction, Residue after removal of solids only"	$1:10^4$	15	16.955	41.9
"Solvent Extraction fraction #152.5 (B158)	$1:10^4$	9	20.810	74.2
"Aqueous Alkali Extract of Whole Creosote	$1:10^4$	15	13.640	14.3
"Creosote with Tar Acids and Tar Bases Removed"	$1:10^4$	18	14.960	25.2
"Creosote with Tar Bases Removed"	$1:10^4$	14	23.920±2.56	100.5

* Oxygen consumption is expressed as mm^3/hr/group of 25 animals
** All determinations were made using twenty-five Limnoria per Warburg flask

It will be observed in Table 1 that "Creosote with Tar Acids and Tar Bases Removed", "Aqueous Alkali Extract of Whole Creosote" and M/1,000 Glucose in sea water are all about equally effective in increasing the oxygen consumption of Limnoria.

On the other hand the two fractions which were produced by solvent extraction produced approximately the same respiratory response as whole creosote at comparable dilution. "Creosote with Tar Bases Removed" was easily the most effective fraction studied, resulting in an increase of over 100% in oxygen uptake over the rate of untreated normals.

These results should be considered to be preliminary in nature. They demonstrate that the method has definite utility in differentiating between creosote preparations. This method should contribute to the problem of fractionation of the toxicity of creosote and will vastly accelerate the assay procedure.

6. Cellulase enzyme system.

This system has been observed to be present and active in the free swimming larvae of Teredo. This observation suggests that cellulytic activity may contribute to penetration of the wood by larval shipworms. Cellulase activity survives in larvae which have been dried in vacuo at 50°C. The activity is only slightly diminished in such preparations. Some cleavage of cellulose is produced even after exposure of the incubation mixture to temperatures in the neighborhood of 90°C for a period of five minutes. An attempt has been made further to purify the active principle in homogenates of adult shipworms. A fraction of high activity and somewhat reduced enzymatic contamination is produced by ad-

sorption of cell-free homogenate on a precipitated form of carboxy-methyl-cellulose (Hercules Powder Co.) After washing the adsorption complex free of soluble and insoluble organic material, the enzyme is then liberated from the cellulose by dissolving the latter. Fractional precipitation of the resulting solution has produced the cellulytically active material. Further studies are being conducted to determine exact degree of concentration of the activity, and the relation between activity and total nitrogen content of the purified preparation. The activity of the shipworm cellulase is also being studied in relation to the activity of the standard mold enzyme produced by Myrothecium verrucaria.

7. Carbohydrate metabolism

Previous studies on glycogen content and anoxia in the shipworm have led to the present investigation of the organism's carbohydrate metabolism - especially that associated with the anaerobic phase. The measurement of energy consumption under these conditions involves quantitative analysis of certain of the glycolytic intermediate compounds coupled with studies of oxygen uptake during the terminal stages of glycolysis.

A qualitative analysis of some of the intermediates was accomplished by running paper chromatograms of shipworms homogenates. The organisms were extracted from the infected wood and chilled immediately. The soft tissues were then homogenized and centrifuged. Chromatograms were set up using drops of supernatant in the method of Eggleston and Hems (Biochem. J., 52, 1, 156-160, 1952). In this manner, the following substances were detected: adenylic acid, adenosine polyphosphates (ATP & ADP), hexose

phosphates, and arginine phosphate.

Due to the high concentration of arginine phosphate in the teredo, this substance was selected from the above group for quantitative analysis to be correlated with further anaerobic studies. Using the method of Brand and Kassell (J. Biol. Chem., 145, 2, 359-364, 1942) the following results were obtained:

	Body Fluid Arginine			Tissue-bound Arginine	No Animals
Species	Phospho arginine Range % Dry Wt.	Free Arginine Range % Dry Wt.	Total Arginine % Dry Wt. Average	% Dry Wt. Average	
Teredo bipartita.	0.0490-0.120	0.222-0.291	0.339	0.858	28
Teredo bartschi	0.0182-0.186	0.0851-0.244	0.272	0.901	31

Continued studies are in progress which involve the measurement of increasing lactate and pyruvate under anaerobic conditions. When the organisms have reached the state of maximum concentrations of these substances, they will be re-exposed to an oxygenated environment, and their recovery and oxygen uptake will be measured.

8. Larval Histology

Micro-technique equipment for the study of larval histology has been set up in anticipation of the expected fall outburst of larvae. A large tank has been set up in the Virginia Key Laboratory to receive shipworm infested blocks which have been exposed in the bay for several months. An all plastic, non-toxic, trap has been constructed to catch

the larvae. At the present time a literature survey is also underway.

9. X-ray Studies on Growth

Work on this aspect of the general problem has been substantially completed. The results are being prepared for publication as a technical report.

10. Pre-Soaking Tests

Tests to determine the effect of prior immersion on attack were initiated in April. Identical fir panels, 2" x 6" x 3/4", were soaked in sterile filtered fresh water, sterile filtered sea water, and filtered bay water for periods of 1, 2, 4, 8, 16, and 32 days. The periods of immersion of the panels were so spaced that they terminated on the same day. They were transferred, underwater, to the exposure rack so that at no time were any of the panels exposed to aerial bacteria. Unsoaked control panels from the same fir stock were attached to the rack at the same time. The panels were exposed on 13 June 1953.

No attack was noted until 6 July 1953. At that time three small Limnoria burrows were found. The panels attacked first were a control panel, a panel exposed for two days in sterile fresh water, and a panel exposed four days in sterile salt water. Each panel had a single small Limnoria burrow. At the end of one month a second control showed a single Limnoria burrow as well as a shipworm burrow. The panel exposed for 32 days in sterile salt water showed similar attack.

At the end of two months exposure all of the panels showed very slight Limnoria attack. Every panel, similarly, showed slight Teredo attack with a count of burrows ranging from 4 to 10 with no definite

alignment noticeable as can be seen in the table.

Panel	Limnoria	Teredo	
Control. Unsoaked	1	1	5 holes
Control. Unsoaked	1	1	4 "
Control. Unsoaked	1	1	6 "
1 day in filtered bay water	1	1	5 "
1 day in sterile salt water	1	1	6 "
1 day in sterile fresh water	1	1	4 "
2 days in filtered bay water	1	2	8 "
2 days in sterile salt water	1	1	6 "
2 days in sterile fresh water	1	1	5 "
4 days in filtered bay water	1	1	4 "
4 days in sterile salt water	1	1	4 "
4 days in sterile fresh water	1	2	8 "
8 days in filtered bay water	1	2	10 "
8 days in sterile salt water	1	1	6 "
8 days in sterile fresh water	1	1	6 "
16 days in filtered bay water	1	1	4 "
16 days in sterile salt water	1	1	5 "
16 days in sterile fresh water	1	1	4 "
32 days in filtered bay water	1	1	6 "
32 days in sterile salt water	1	1	5 "
32 days in sterile fresh water	1	2	10 "

11. Chlorinated Creosote Panel Exposure Tests

Panels impregnated with chlorinated creosote prepared by NRL were exposed 28 January 1953. Inspection at the end of four months showed only very limited attack.

12. Toxic Diffusion Tests

Holiday panels were exposed on 13 November 1952. Inspection on 13 August showed the following:

Size of Holiday	Creosote		Damage to treated and untreated areas: Antifouling Paint		White Deck Paint	
	Treated	Untreated	Treated	Untreated	Treated	Untreated
1/8"	F-3 L-0 T-0	F-3 L-4 T-4	F-1 L-0 T-0	F-1 L-0 T-0	F-5 L-5 T-5	F-5 L-5 T-5
1/4"	F-3 L-0 T-0	F-3 L-5 T-?	F-1 L-0 T-0	F-1 L-0 T-0	F-5 L-5 T-5	F-5 L-5 T-5
1/2"	F-3 L-0 T-0	F-3 L-5 T-?	F-0 L-0 T-0	F-1 L-0 T-0	F-5 L-5 T-5	F-5 L-5 T-5

The creosoted panels and those treated with deck paint were removed from the water on this date. It is apparent that antifouling paint effectively protects adjacent holidays of as much as ½". Creosote has barely appreciable effect at 1/8".

Unleached creosoted blocks serving as a long term field test for accelerated leaching tests were exposed 24 April 1951. Inspection on 30 July 1953 showed the following:

1" x 2" block (30.7 lbs/ft^3)	Fouling	Very heavy	- 5
	Limnoria	No attack	- 0
	Teredo	No attack	- 0
2" x 4" block (26.6 lbs/ft^3)	Fouling	Very heavy	- 5
	Limnoria	No attack	- 0
	Teredo	No attack	- 0

13. Tropical Timbers

A block of Greenheart (Ocotea rodiaei) was exposed 1 June 1950. Inspection on 8 July 1953 showed the following, after 3 years exposure:

Fouling	Very heavy	- 5
Limnoria	A few small burrows, very light attack	- 1
Teredo	Several medium to large burrows plus scattered pits and very young burrows	- 3

Silica-containing Surinam timber panels (1" x 4" x 12") were exposed 7 February 1951. Inspection on 12 August 1953 showed the following:

Panel No.	Local Name	Fouling	Limnoria	Teredo
1107	Bongro Foengoe	Lost May 1952, no attack		
1113	Jan Snijder	" "	" " "	
1110	Anaura	" "	" " "	
1111	Anaura	3	0	1
199a	Savanna Foengoe	3	3	3
1106	Foengoe	Lost May 1952, no attack		
197	Man Foengoe	Lost Feb. 1953, light attack		
1115	Sopohoedoe	Destroyed by Teredo, June 1952		
198	Zwarte Rienhout	3	1	3
1102	Witte Rienhoat	3	4	4
131	Manbarklak	3	1	1

Note: Panel attack is numerically rated according to the following table:

0 No attack
1 Very light attack
2 Light attack
3 Moderate attack
4 Heavy attack
5 Very heavy attack, riddled

14. Facilities

At the beginning of August, a new laboratory building on the seashore at Virginia Key was completed and occupied. This provides continuous adequate seawater circulation for experimental purposes. The laboratory rearing of Teredo and Limnoria has been transferred to this station together with physiological operations which require the use of supplies of living material. The test exposures continue to be carried out at Miami Beach where the incidence of borer attack is at a maximum.

15. Publications:

GREENFIELD, LEONARD J. Observations on the Nitrogen and Glycogen content of Teredo (Lyrodus) pedicellata de Quatrefages at Miami, Florida. Bulletin Marine Science Gulf and Carribbean, Volume 2, No. 3

ISHAM, L. B. and TIERNEY, J. Q. Some aspects of the Larval development and Metamorpheses of Teredo (Lyrodus) pedicellata de Quatrefages. Ibid. Volume 2, No. 4

LANE, CHARLES E. Cellulose Digestion in Teredo. Journal of Biological Chemistry. (In press)

Reprint of the Marine Borer Conference, June 19, 1950. (Expenses carried by the University)